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Monocyte–macrophage differentiation in three dimensional collagen lattice

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Abstract

Human peripheral blood mononuclear cells (PBMC) upon transendothelial migration interact with subendothelial matrix components and differentiate into macrophages. In order to study whether the shape of the cells as dictated by the extracellular matrix can influence monocyte–macrophage (mo–mϕ) differentiation, human PBMC were maintained in vitro on a three dimensional collagen I (COL I) lattice and studied for various macrophage specific functions, viz. endocytosis of [^{125}I]acetyl bovine serum albumin (BSA), expression of specific cell surface antigens and expression of matrix metalloproteinases (MMPs). The cells maintained in three dimensional COL gel exhibited a higher rate of endocytosis of [^{125}I]acetyl BSA than those on COL-coated plastic. FACS analysis showed that the mean fluorescence intensity (MFI) corresponding to monocyte specific LPS receptor CD14 was significantly decreased while MFI corresponding to macrophage specific transferrin receptor CD71 was significantly increased in cells maintained in vitro on three dimensional COL gel compared to two dimensional COL substrata. Expression of macrophage specific MMPs (gelatinase A and gelatinase B) was significantly high in cells maintained on COL gel than on COL I-coated plastic. Appearance of 67 kDa gelatinase in the COL gel suggested that induction as well as activation of MMPs occur when cells are maintained in a three dimensional environment. These results indicate that monocytes undergo a rapid rate of differentiation when maintained in vitro on three dimensional COL I lattice suggesting that apart from the chemical nature of the matrix, the shape of the cells as provided by the matrix also influences mo–mϕ differentiation.

Keywords: Collagen gel; Matrix metalloproteinase; Monocyte–macrophage; Cell surface antigen (CD14 and CD71); Endocytosis

1. Introduction

Cellular interactions with extracellular matrix (ECM) components are crucial for maintaining structural integrity, morphology and signalling processes [1]. In vitro studies using fibroblasts [2], hepatocytes [3–5] smooth muscle cells [6] and mammary epithelial cells [7] maintained on laminin (LN), fibronectin (FN), collagen (COL) or tissue biomatrix and reconstituted basement membrane showed that matrix substrata influences various biochemical and differentiated functions. Individual components of the matrix have been shown to interact with cells through specific matrix receptors causing generation of signals [8] to modulate various intracellular activities in a specific manner. Matrix responsive elements in genes have also been identified [9]. Another possible factor contributing to modulation of cellular activity by matrix is the shape of the cell. It has also been

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demonstrated that mechanical properties of the ECM are critical in regulating cellular actin cytoskeletal organization [10] and signalling [11]. It appears that the shape of the cell as determined by the nature of the matrix substratum influences various biochemical activities [4]. Basement membrane matrigels have been reported to promote mammary gland differentiation [7], cell migration [12] and osteogenesis [13] in vitro.

Monocytes during their transendothelial migration encounter with subendothelial matrix and differentiate into macrophages [14]. Macrophages are involved in the clearance of unphysiologic proteins through the involvement of scavenger receptors [15]. They also secrete growth factors and cytokines and chemoattractants which facilitate further recruitment of monocytes and smooth muscle cell proliferation [16]. An up regulation of the expression of matrix metalloproteinase (MMP) 9 in HL-525 cells maintained on FN suggested that expression of macrophage specific functions is influenced by the nature of the matrix protein [17]. ECM mediates its effect through integrin receptors and the downstream signalling appears to occur via tyrosine kinase dependent phosphorylation as well as microfilament reorganization [8,18,19]. We have recently found that matrix substrata modulate monocyte–macrophage (mo–mϕ) differentiation in vitro. Matrix effect on mo–mϕ differentiation may also be due to the effect on the shape of the cells. In order to examine how far the cell shape as influenced by the subendothelial matrix in vivo can contribute to the expression and maintenance of the differentiated functions of human peripheral blood mononuclear cells (PBMC), we maintained human PBMC on COL I-coated plastic substrata where the cells attach along their basal surface to the basement membrane and on gel forms of COL I where the cells can penetrate into the three dimensional lattice and studied various macrophage specific functions.

2. Materials and methods

2.1. Reagents

Histopaque 1077, RPMI 1640, penicillin, streptomycin, protein markers and gelatin were purchased from M/s Sigma Chem. Co., St. Louis, MO, USA. Fetal calf skin COL I was kindly provided by Jurgen Rauterberg, Münster, Germany. [^{125}I]NaI was a product of BARC (Mumbai, India). Monoclonal antibodies to CD14 and CD71 were purchased from M/s Sigma Chem. Co. PE-conjugated CD14 antibody was obtained from Pharmingen (San Diego, CA, USA). Anti-mouse IgG-FITC was obtained from Santa Cruz Biotechnologies. Cell culture dishes were purchased from Nunc (Denmark).

2.2. Preparation of COL substrata

For three dimensional COL gel, 0.88 ml of COL I in 0.1% acetic acid was mixed with 0.11 ml of 10-fold concentrated RPMI 1640 medium and neutralized with 0.1 ml of 0.34 N NaOH in a plastic culture dish so that the final concentration of COL was 3 mg/ml. The dish was placed in a 37°C incubator until the gel set. Before use, the gels were equilibrated with culture medium. For two dimensional COL substrata, solution containing 50 μg/ml COL I was used for passive coating of the culture dishes as described before [4].

2.3. Monocyte isolation and culture

PBMC were prepared from peripheral blood of healthy donors using Histopaque 1077 as previously described [20]. Cells were collected, washed, resuspended in RPMI medium. Purity of the monocyte preparation as determined by Giemsa stain and immunofluorescence staining using anti-CD14 was more than 95%. Cell preparations of more than 95% viability were used for the experiments. For cell culture, monocytes (1×10^5 cells/ml) were suspended in RPMI medium supplemented with penicillin (100 U/l) streptomycin (100 mg/l) and 5% FN free homologous serum and maintained in culture on COL I gel and plastic coated with COL I substrata under 5% CO_2 atmosphere at 37°C in a Forma incubator. Cells maintained on the COL I gel were collected by 0.2% collagenase treatment as described before [4] while those on COL I-coated plastic were dislodged by treatment with 2 mM EDTA in phosphate-buffered saline at 4°C and subsequently phenotypic as well as functional characteristics of monocyte-derived macrophages were analyzed.

2.4. Endocytosis

Endocytosis of modified protein was studied by supplementing the culture medium with radioiodinated acetyl bovine serum albumin (BSA) for 6 h at 37°C. Acetylation and radioiodination of BSA were done as previously described [21,22]. The cellular uptake (acid precipitable) and degradation (acid soluble) of [^{125}I]acetyl BSA were determined by precipitation with 10% trichloroacetic acid and radioactivity was measured in an LKB minigamma counter. Protein was estimated by the method of Lowry et al. [23].

2.5. Zymography

Activity of MMPs in medium secreted from cells maintained on COL I gel and COL I-coated plastic was determined by preparing zymograms [24]. Zymogram gels consisted of 7.5% polyacrylamide gel copolymerized with gelatin (1 mg/ml). For total gelatinase activity, multiwell zymography was done where the substrate gel was copolymerized with the culture medium, followed by incubation with substrate buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 0.02% NaN_3, pH 7.5) at 37°C. Gels were stained with Coomassie blue R250 and destained with water. Gelatinolytic activity, appeared as clear zones, was quantified using an LKB ultroscan laser densitometer.

2.6. Flow cytometric analysis

PBMC were maintained in culture on COL I gel and COL I-coated plastic for 4 h and 96 h. The cells were dislodged and non-specific binding through Fc receptor was blocked using 1% goat serum (Dako, Golstrup). The cells were then processed for dual staining for surface markers CD14/CD71 by treating cells first with antibody to CD71 followed by appropriate FITC-labelled secondary antibody for 30 min at 4°C. Cells were then fixed with paraformaldehyde for 5 min at 4°C and treated with PE-labelled CD14 antibody for 30 min at 4°C. The cells were also stained with corresponding FITC- or PE-conjugated isotype-matched control mouse IgG (Becton Dickinson) as negative controls. Fluorescence was determined by FACS Vantage using a 488 nm argon laser (Becton Dickinson, Mountain View, CA, USA). For acquisition of data gated cell populations were employed. Measurement of mean fluorescence intensity (MFI) and analysis of data were done using Cell Quest Software (Becton Dickinson).

3. Results

PBMC plated on two dimensional COL-coated substrata spread and developed a flat and extended morphology whereas cells maintained on COL I gel appeared to penetrate the three dimensional substratum and maintain a morphology similar to that in vivo.

3.1. Endocytosis of [^{125}I]acetyl BSA

Differentiation of monocyte to macrophage is marked by the ability of the cells to endocytose unphysiologic proteins through the involvement of scavenger receptors. To study endocytosis, culture medium was supplemented with [^{125}I]acetyl BSA

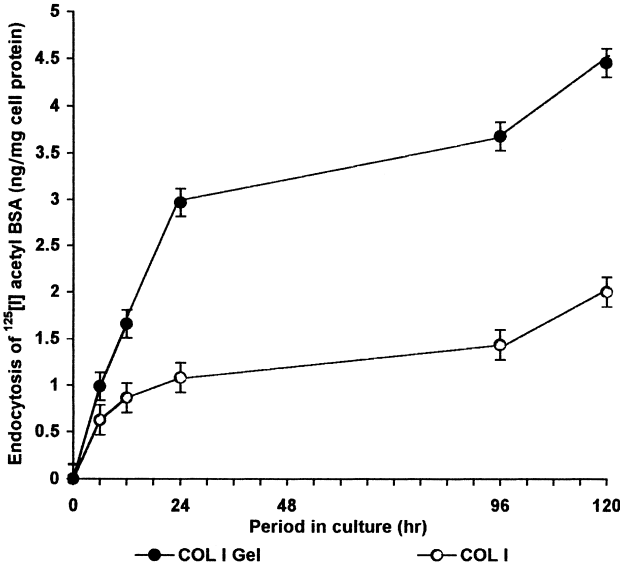

Fig. 1. Endocytosis of [^{125}I]acetyl BSA by monocytes maintained in culture on COL I gel. Mononuclear cells (1×10^5 cells/ml) were maintained in vitro on COL I gel (●) and COL I (○)-coated plastic for different time intervals. Cells were supplemented with serum free medium containing [^{125}I]acetyl BSA (2 μg/ml) on each day and the amount of protein endocytosed at 37°C in 6 h was measured as described in the text. Results are expressed as mean ± S.D. of two independent experiments done in triplicate ($P < 0.01$).

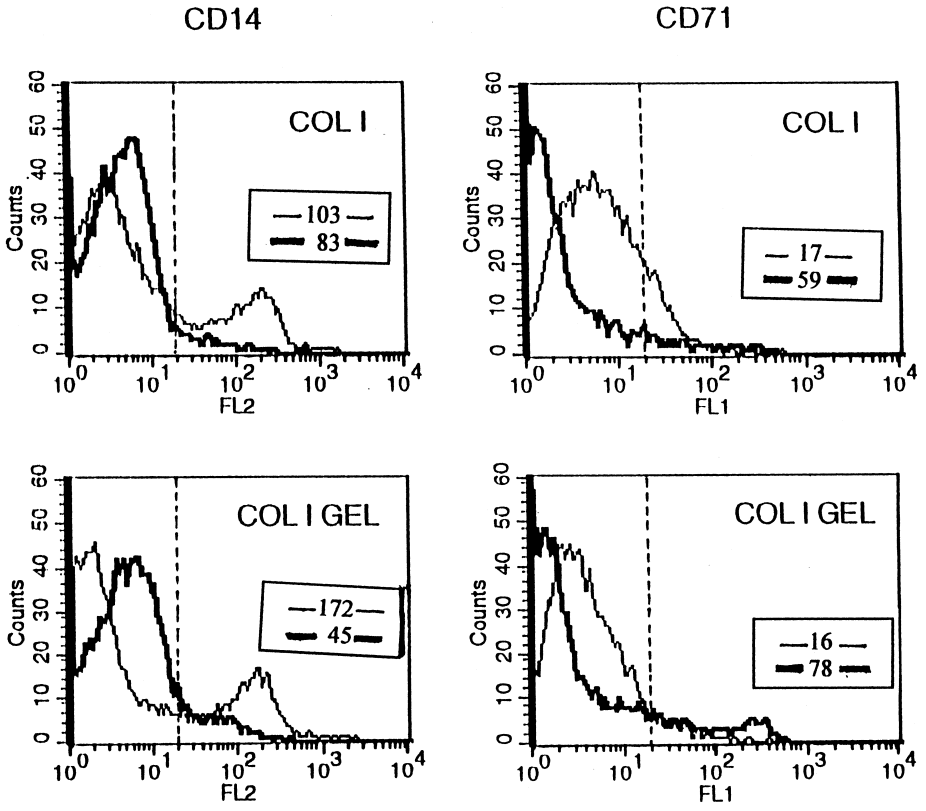

Fig. 2. Expression of CD14 and CD71 on mo–mϕ maintained on COL I gel. Mononuclear cells (1×10^5 cells/ml) were maintained in vitro on COL I and COL I gel for 4 h and 96 h. Cell surface expression of CD14 and CD71 was assessed using monoclonal antibodies to CD14 and CD71 using FACS. The fluorescent histograms of a representative experiment of three are shown. Thin line represents 4 h and thick line represents 96 h. In insets are given the MFI values corresponding to the fluorescent histograms.

and its uptake by cells during a period of 6 h was studied. The ability of monocytes maintained on COL I gel and COL I-coated plastic to endocytose [^{125}I]acetyl BSA progressively increased with the duration of culture. The amount of [^{125}I]acetyl BSA endocytosed by cells maintained on COL gel was significantly greater than those maintained on COL I-coated plastic substrata at all time intervals tested (Fig. 1).

3.2. Surface antigen expression

The ECM organization in a three dimensional matrix would mimic the in vivo environment and thus maintain a functional mo–mϕ phenotype. Cell surface antigen expression of monocyte specific CD14 and that of macrophage specific CD71 were assessed on cells maintained on COL I gel as well as COL I-coated plastic. These markers were analyzed at 4 h and 96 h of culture using FACS. Cells maintained on COL I gel as well as COL I-coated plastic for 4 h were positive for CD14 and negative for CD71. At 96 h, there appeared an up regulation of CD71 antigen with a down regulation of CD14. Decrease in the MFI corresponding to CD14 at 96 h was significantly more in cells maintained on COL I gel than those on COL I-coated plastic. MFI corresponding to CD71 after 96 h was significantly greater in cells maintained on COL I gel than those on COL I-coated two dimensional substrata (Fig. 2).

3.3. Induction of MMPs

MMPs are secreted by mononuclear phagocytes which participate in matrix turn over as well as cell migration. In the present study, the influence of COL I gel substratum on the induction of gelatinase expression was studied by zymography of medium secreted by cells maintained on COL I gel. Freshly isolated monocytes did not exhibit gelatinase activity.

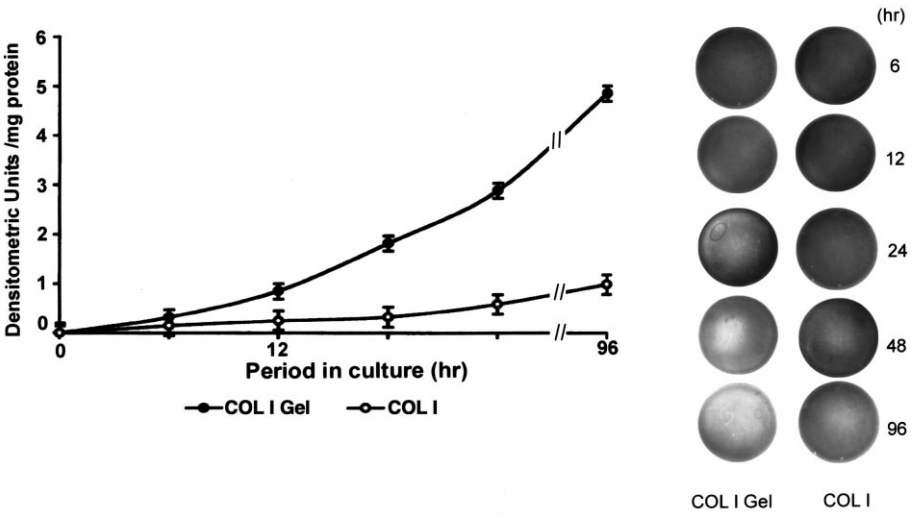

Fig. 3. Expression of total gelatinase by mo–mϕ maintained in vitro on COL I gel. Mononuclear cells (1×10^5 cells/ml) were maintained in vitro on COL I gel (●) and COL I (○)-coated multiwells and the total gelatinase activity secreted into the medium during different periods of culture was assessed by doing zymography in multiwells. Gelatinolytic activity was measured densitometrically and the activity was expressed as densitometric units/mg protein. Results are expressed as mean ± S.D. of two independent experiments done in triplicate. In inset is given zymography done in multiwells.

Gelatin zymography performed in multiwells for total gelatinase activity showed that there was significant increase in gelatinolytic activity secreted by cells maintained on three dimensional COL I matrix substrata. Kinetic analysis revealed that within 24 h itself, cells maintained on COL I gel secreted gelatinase into the medium while those on COL I-coated plastic did not produce any significant amount of gelatinase during this period (Fig. 3). This was further analyzed by doing zymography in gelatin containing polyacrylamide gel which demonstrated the production of two major bands of 92 kDa and 72 kDa (Fig. 4). With the progression of culture, another gelatinolytic proteinase of ≅67 kDa also appeared in the culture medium of cells on COL I gel while this was completely absent in medium of cells maintained on two dimensional COL I substrata for the same period of culture. Immunoblot analysis showed that 92 kDa gelatinase was MMP 9 and 72 kDa gelatinase was MMP 2 (data not shown). The 67 kDa appeared to be the activated form of 72 kDa. The activities of these individual MMPs produced by cells maintained on COL I gel were significantly higher than those in the medium of cells maintained on COL I-coated plastic. However the total gelatinase activity produced by cells maintained on COL I gel appeared to be more than the sum of the activities of the individual MMPs identified by zymography.

4. Discussion

In vitro studies with different cell systems showed that ECM, which is an intricate assembly of proteins, viz. COL, LN and FN [25], influences various cellular activities and differentiation [2–6]. Cell–ECM interactions play a major role in regulating and maintaining connective tissue integrity [26]. Monocytes during their egress from circulation encounter with subendothelial matrix components and differentiate into tissue macrophages [14]. Studies using human PBMC maintained in vitro on different ECM components showed that mo–mϕ differentiation varies as the chemical nature of the matrix substratum is altered; for instance, FN was found to be more effective than COL in this process (unpublished data). Apart from the chemical nature, one of the factors crucial in regulating the cell behavior is the shape of the cell as provided by the matrix. Morphological analysis done by electron microscopy showed that cells adhering to different matrix proteins acquire different shapes which in turn depend on the actin cytoskeletal organization [27]. An in vitro model sys-

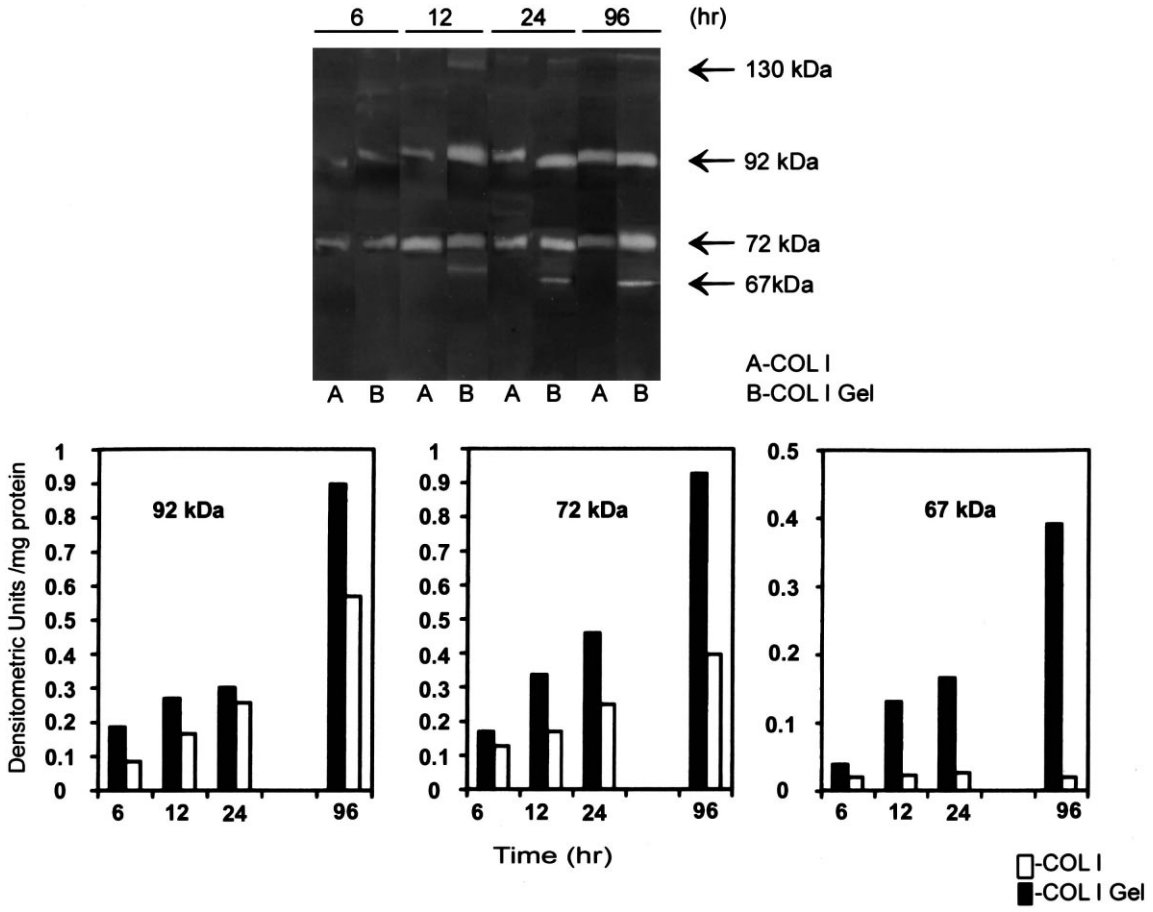

Fig. 4. Expression of individual MMPs by mo–mϕ maintained in vitro on COL I gel. Mononuclear cells (1×10^5 cells/ml) were maintained in vitro on COL I gel (■) and COL I (□)-coated multiwells and the individual MMP activity secreted into the medium during different periods of culture was assessed by doing zymography in 7.5% minigels. Gelatinolytic activity was measured densitometrically and the activity was expressed as densitometric units/mg protein. Results are expressed as mean of two independent experiments done in triplicate. Upper panels shows zymography done in minigels.

tem of PBMC maintained on three dimensional COL lattice where the cells can adhere and penetrate into the COL fiber to mimic the in vivo status is used to study mo–mϕ differentiation. Being the most abundant component of the vertebrate connective tissue, COL was used as the substratum. It appears from our studies that monocytes undergo a rapid rate of differentiation to macrophages when maintained on three dimensional COL gel than on two dimensional COL substrata. The evidence in support of this is a higher rate of appearance of macrophage specific functions such as (i) endocytosis of modified protein, (ii) expression of a phenotype characteristic to macrophage as well as (iii) induction and activation of MMPs.

Alterations in cell shape are mediated by intracellular assemblies [28] and are influenced by both the chemical [29] and physical nature of the substratum onto which the cells adhere. Earlier reports suggested that regulation of differentiated functions by ECM may be due to the altered morphology [30]. Our data suggest that the rate of differentiation of mo–mϕ is different when the cells are allowed to assume different contours and appear more similar to the morphology in vivo.

Although a greater surface area of cells maintained in three dimensional COL gel can contribute to higher endocytosis, a progressive increase in the relative rate of uptake of modified protein suggests the involvement of other factors. Endocytosis of unphysiologic proteins is mediated through the involvement of scavenger receptors, viz. SR-A [31], SR-B and CD36

[32]. An up regulation/activation of scavenger receptors occurring in cells maintained on COL I gel can contribute to increased endocytosis. There are also indications that the influence of ECM on the phenotypic expression of cells may be due to its effect on cell shape and on the specific cytoskeletal patterns [30,33]. The phenotypic changes observed in the present study on monocyte maintained on COL I gel suggest that the shape of the cells as provided by the ECM can influence changes in the cell surface antigen expression.

Remodeling of ECM is an important event during many biological and patho-physiological processes and is brought about by a family of cation dependent MMPs. One of the characteristic changes that occur during mo–mϕ differentiation is a marked increase in the expression of MMP genes [34,35]. Disassembly of old and deposition of new ECM components have to be tightly regulated both spatially and temporally. Production of MMPs as assessed by gelatin zymography of medium secreted by cells grown on COL I gel in our present study showed that the total MMP activity was about 4–5-fold greater compared to that of two dimensional matrix substrata. An analysis of the relative amounts of various MMPs produced by mo–mϕ revealed that the major MMPs are 92 kDa gelatinase B and 72 kDa gelatinase A. Apart from an increased production of these major MMPs, significant activation of 72 kDa to 67 kDa also occurs when cells are in three dimensional gel. Although the sum of the activities of the individual enzymes accounts for about 90% of the total gelatinase activity produced by cells in two dimensional supports in the case of cells maintained on COL I gel, the activities constituted only about 50% of the total (Figs. 3 and 4). This may be due to either non-detection of any new form of MMPs on zymogram, or due to a limitation of the assay at a higher concentration of the enzymes or due to both.

An increase in the activity of 67 kDa, the activated form of MMP 2, in the medium of cells maintained on COL gel suggests a faster rate of activation of MMPs in a cell morphology dependent manner. Tomasek et al. demonstrated that fibroblasts expressed and activated MMPs only in a three dimensional COL culture [36–38]. It therefore appeared that the transcription of MMP genes was sensitive to the alterations in the physical state rather than to COL ligand itself. We found that cells retained on two dimensional matrix substrata did not produce the active form of MMP 2 (unpublished data). Oliver et al. showed that transducing information in a three dimensional structure depends on a three dimensional conformation of relevant receptors and/or its ligand [11]. Interaction of cells with such a COL lattice may result in a cytoskeletal reorganization which can probably induce changes in the expression of differentiated functions. Integrin-mediated signalling is reported to be crucial for MMP gene expression [11,39,40], which is a macrophage specific function. This in vitro model system can be useful in examining how integrins transduce information from three dimensional contact of matrix protein into cellular events in monocytes.

The present study demonstrated that mo–mϕ differentiation as well as acquisition of macrophage specific functions is dependent on the shape of the cells as determined by the ECM components with which they are in contact. Further investigations utilizing this in vitro model would permit identification of mechanical signals from ECM regulating cell behavior which are important in normal physiological as well as patho-physiological conditions like atherosclerosis, diabetes and aging.

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